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Excitatory Amino Acids Contribute to the Pathogenesis of Perinatal Hypoxic-Ischemic Brain Injury

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A large body of experimental evidence indicates that over-activation of excitatory amino acid (EAA) receptors may mediate irreversible neuronal injury in a variety of pathologic settings including cerebral ischemia, and that the developing brain may be particularly susceptible to the adverse effects of EAA receptor overactivation. In this article, we review current information about EAA receptor pharmacology and EAA neurotoxicity in the immature brain, and summarize recent experimental data indicating that EAA contribute to the pathogenesis of perinatal hypoxic-ischemic brain injury.

Introduction

In the last decade, several seminal observations have provided support for the hypothesis that endogenous excitatory amino acid (EAA) neurotransmitters (e.g., glutamic acid) play a major role in the pathgenesis of hypoxic-ischemic neuronal injury. *In vitro*, in cultured hippocampal neurons, Rothman first demonstrated that anoxic neuronal injury was dependent on synaptic activity; a substantial body of subsequent work confirmed that EAA mediated anoxic neuronal injury *in vitro* (1). Concurrently, in experimental animal models of ischemic brain injury, a variety of experimental strategies yielded complementary evidence that EAA contributed to the pathogenesis of irreversible ischemic neuronal injury. Important findings included: the recognition that regions with high densities of EAA receptors were particularly susceptible to ischemic injury (2), observations that intra-cerebral injection of the competitive EAA antagonist arninophosphonoheptanoic acid (AP7) attenuated acute ischemic neuronal damage (3) and that preceding deafferentation reduced ischemic damage to highly vulnerable hipppocampal neurons (4), and results of *in vivo* microdialysis studies that demonstrated directly substantial increases in hippocampal extracellular glutamate concentrations during cerebral ischemia (5). In the immature nervous system, Hagberg et al. demonstrated that in fetal lambs, cerebral ischemia resulted in marked rises in cortical and striatal glutamate content (6) and McDonald et al. showed that treatment with the noncompetitive EAA antagonist MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo-(a,d)-cycloheptene-5,10-imine maleate] was neuroprotective in a model of perinatal focal cerebral ischemia (7).

Current important experimental questions include elucidation of the distinct roles of EAA in the pathogenesis of neuronal injury after focal and global ischemia, development of effective neuroprotective interventions based on EAA pharmacology (8,9), and understanding the potential adverse effects of drugs acting at EAA receptors. In this review, we will present results of recent studies that provide important new insights about the potential contributions of EAA to perinatal hypoxic-ischemic brain injury.

Excitatory Amino Acid (EAA) Pharmacology

An understanding of EAA neuropharmacology is an essential prerequisite for analysis of the role of EAA in the pathogenesis of brain injury and for development of therapeutic strategies based on EAA receptor blockade. In theory, it would be possible to modulate EAA synaptic function by altering synthesis, release, re-uptake of EAA, or receptor activation. Few pharmacologic agents with specific pre-synaptic actions

Glossary for Abbreviations	
EAA	Excitatory amino acids
PND	Post-natal day
ECF	Extracellular fluid
HPLC	High performance liquid chromatagraphy

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have been developed, and EAA pharmacology is currently focused primarily on analysis of post-synaptic receptors.

Several classsification schemes for EAA receptors have been devised, based on their selective responses to the agonists N-methyl-D-aspartate (NMDA), quisqualic acid and kainate, and on their signal transduction mechanisms (ionotropic or metabotropic) (10-12).

NMDA receptor-channel complex. The NMDA-receptor channel complex includes the EAA recognition site, a voltage-dependent cation channel permeable to both sodium and calcium, which, at rest, is blocked by magnesium, and a strychnine-insensitive glycine receptor that is activated by physiologic concentrations of glycine. Several distinct groups of NMDA antagonists, with different loci of action, have been identified (13). The NMDA receptor-channel complex can be blocked competitively at its agonist binding site, by compounds such as aminophosphonoheptanoic acid (see Table). Non-competitive antagonists such as phencyclidine (PCP) and MK-801 [(+)-5-methyl-10, 11-dihydro-5H-dibenzo (a,d) cyclohepten-5-10-imine maleate] bind at sites within the ion channel, and glycine antagonists (e.g., 7-chlorokynurenate) can also functionally block NMDA receptor activation (see Table). Distinct binding sites for polyamines have also been identified, and polyamines appear to be have complex regulatory influences on NMDA-receptor function (14).

Many recent studies have evaluated the neuroprotective efficacy of competitive and non-competitive NMDA antagonists in experimental animal models of ischemic brain injury. Numerous reports have demonstrated efficacy in models of focal cerebral ischemia (15-17), however, other studies attributed beneficial effects to non-EAA-mediated effects (18). Furthermore, in models of global cerebral ischemia, generally, NMDA antagonists have not proven to be effective neuroprotective agents (19).

Another important observation relevant to the potential therapeutic actions of NMDA antagonists is their potential deleterious effects (20); Olney and colleagues first reported that MK-801 induced acute vacuolar degeneration in selected cell populations and subsequently showed that these degenerative changes could be prevented by concurrent administration of anti-cholinergic drugs or diazepam (21).

Non-NMDA receptors. There are two groups of quisqualate-sensitive receptors, those linked with a cation channel and selectively responsive to the agonist alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and the metabotropic quisqualate receptor (linked with phospholipase C). A distinct group of EAA receptors respond selectively to kainic acid. The ionotropic receptors are linked with cation channels permeable to sodium. Effective AMPA receptor antagonists have also recently been developed. The systemically administered AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) is neuroprotective in models of global cerebral ischemia in adult gerbils and rats (22-24).

Recent advances in molecular analysis of EAA receptor encoding genes suggest that there may be considerably greater diversity in EAA receptor expression than is currently discernible pharmacologically. Multiple genes encoding EAA receptors or constituent subunits have been cloned, and ontogenetic changes in the regional distributions of the mRNAs for specific EAA receptor subunits have been identified (25).

EAA-Mediated Neurotoxicity

A large body of experimental data indicates that over-activation of EAA receptors by endogenous or exogenous agonists can cause neuronal damage (the concept of "excitotoxicity") (26). Increased activation of EAA receptors could reflect elevated synaptic concentrations of EAA (because of increased release or failure of neuronal or glial re-uptake) or increased receptor sensitivity to the agonist.

Molecular mechanisms of neurotoxicity. The molecular mechanisms that mediate EAA-induced injury have, for the most part, been elucidated *in vitro*. Current evidence indicates that overwhelming increases in free intracellular calcium (attributable to increased entry through NMDA and voltage-gated channels and inability of mitochondria to compensate) represent the final common pathway of EAA-mediated injury (27). The blockade of NMDA-induced calcium entry by the neuroprotective non-competitive antagonist MK-801 has recently been documented *in vivo* (28).

Relevant to understanding EAA-mediated mechanisms in ischemic injury is the observation that the vulnerability of cultured neurons to glutamate neurotoxicity rises during glucose deprivation, anoxia or pharmacologic inhibition of oxidative phosphorylation (29), perhaps because of limited adaptive capacity of metabolically compromised mitochondria to rises in free intracellular calcium. In fact, the relationship between compromised energy state and susceptibility to EAA-mediated injury may be relevant to the pathogenesis of irreversible injury in a variety of pathologic conditions (for review, see Ref. 30).

Excessive EAA-receptor activation may have other potentially deleterious effects as well. In some cell populations, EAA's activate phospholipase A2, and stimulate release of arachidonic acid (31). Arachidonic acid has many potential deleterious effects on cell membranes; in addition, it may directly suppress neuronal and glial EAA re-uptake mechanisms and thereby contribute to synaptic EAA accumulation

Table 1 Drugs acting at excitatory amino acids (EAA) synapses

Receptor/Site	*Agonists*	*Antagonists*
NMDA		
EAA recognition	Glutamate, NMDA	AP-5, AP-7, CPP, CGS-19755
Ion channel	Unknown	MK-801, PCP, TCP, dextromethorphan, ketamine
Glycine site	Glycine, D-serine	Kynurenate, 7-chlorokynurenate, HA-966, CNQX, DNQX
AMPA	Glutamate, AMPA, quisqualate, kainate, ibotenate	NBQX, CNQX, DNQX
Metabotropic	Glutamate, quisqualate, trans-ACPD, ibotenate	Aminophosphonoproprionic acid
Kainate	Glutamate, kainate, domoate	CNQX, DNQX, kynurenate

Abbreviations

AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate
APS	D(-)-2-amino-5-phosphonovalerate
AP7	D(-)-2-amino-7-phosphonoheptanoate
CPP	3 (-)-2-carboxypiperazin-4-ylpropyl-1-phosphonate
CGS- 19755	cis-4-phosphonomethyl-2-piperidine carboxylate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
DNQX	6,7-dinitroquinoxaline-2,3-dione
HA-966	1-hydroxy-3-aminopyrrolid-2-one
MK-801	(+)-5-methyl-10,11 l-dihydro-5H-dibenzo-[a,d]-cyclohepten-5,10-imine maleate
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline
NMDA	N-methyl-D-aspartate
PCP	Phencyclidine
TCP	1-[1-(2-thienyl)-cyclohexyl]piperindine
Trans-ACPD	Trans-1-aminocyclopentane-1,3-dicarboxylate

(32). Recent data suggest that NMDA neurotoxicity may also be mediated by activation of ornithine decarboxylase, which results in increased polyamine synthesis. The polyamine synthesis inhibitor alpha-difluoromethylornithine blocked NMDA-induced neurotoxicity *in vitro* (33) and *in vivo* (34). EAA may also stimulate formation of nitric oxide (NO) (35); both *in vitro* and *in vivo* data suggest that NO may be neurotoxic (36). Treatment with N^G-Nitro-L-arginine, a compound that blocks NO synthesis, limited the severity of injury in an adult rodent model of focal ischemic brain injury (37).

EAA neurotoxicity in the developing brain. Immature neurons may be particularly sensitive to the deleterious effects of EAA receptor overactivation. The ontogeny of excitotoxicity has been studied by histopathologic assessment of brain injury after direct intra-cerebral injections of agonists at different developmental stages. In contrast with the resistance of the immature mammalian brain to kainic acid, NMDA neurotoxicity peaks in the early post-natal period. The neurotoxicity of NMDA in hippocampus, striatum and neocortex is maximal in the immature rat brain, peaking at postnatal day six to seven

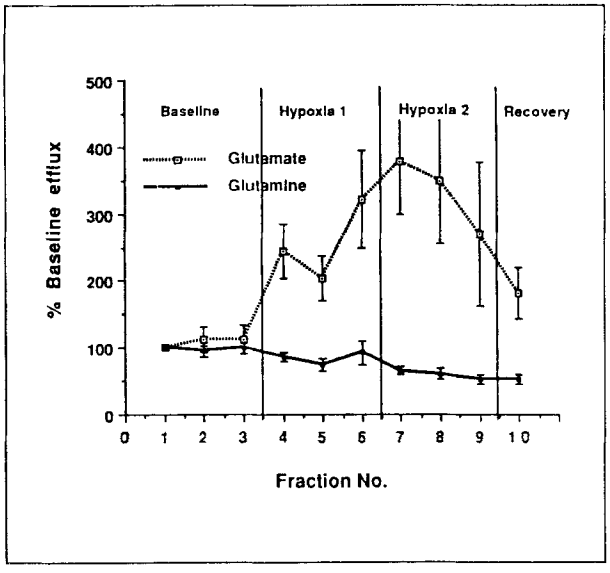

Figure 1 Hippocampal glutamate efflux during perinatal hypoxia-ischemia: Microdialysis probes were inserted into the right hippocampus of PND 7 rats that underwent right carotid ligation, followed by exposure to 8% oxygen for two hours (n=6, see Ref. 56 for methods). Beginning two hours after probe insertion, ten sequential 20 minute dialysis samples were collected from each animal. Amino acid content of dialysates was quantitated by an HPLC assay. This graph compares sequential hippocampal glutamate and glutamine efflux values, expressed as % baseline, in six animals that underwent right carotid artery ligation followed by two hours of 8% oxygen exposure. Baseline values were defined as the averages from the first three fractions collected in each animal. Reproduced from Referece 56, with permission from The International Society for Pediatric Research.

(38,39). Stereotaxic injections of NMDA (5-25 nmol) into posterior striatum produce dose-dependent injury with neuronal necrosis in striatum and overlying dorsal hippocampus and cortex. At this developmental stage, susceptibility to quisqualic acid neurotoxicity is also relatively high, as compared with adult brain (40); yet, NMDA is considerably more toxic (100 nmol quisqualate or 10 nmol NMDA elicit injury of similar severity) (41). Intracerebral injection of glutamate typically results in no injury because of rapid neuronal and glial uptake; yet, injections of massive doses may also produce small brain lesions (42).

Enhanced sensitivity of the immature brain to EAA-induced toxicity could reflect increased receptor density, altered receptor sensitivity (due to age-related differences in the molecular constitution of EAA receptors), or differences in modulatory or compensatory mechanisms. Autoradiographic receptor binding assays indicate that in certain regions (e.g. hippocampus, globus pallidus), the immature brain has a higher density of both NMDA and non-NMDA type EAA receptors than adult brain; however, the ontogenetic peaks of receptor density and susceptibility to neurotoxicity do not coincide precisely (43-46). In electrophysiologic studies, hippocampal pyramidal neurons show maximal responsiveness to NMDA at PND 5 to 9 (47), and immature hippocampal neuron NMDA receptor/channels are less susceptible to blockade by Mg^{++} and more sensitive to modulation by glycine (48,49) or polyamines (14). Alternatively, some populations of immature neurons may be less able to buffer EAA-induced increases in free intracellular calcium, due to immaturity of mechanisms such as the calcium-binding protein calbindin D28k (50).

EAA and Perinatal Hypoxic-Ischemic Brain Injury

Several complementary experimental approaches have been used to examine the role of EAA in the pathogenesis of perinatal hypoxic-ischemic brain injury. An experimental model of perinatal stroke, elicited by unilateral carotid ligation and subsequent timed exposure to moderate hypoxia in immature rats, has been used extensively in these studies (51). In seven day old rats, unilateral carotid ligation followed by exposure to an 8% oxygen/balance nitrogen atmosphere for 2 to 3.5 hours elicits ipsilateral forebrain injury. Neither ligation alone or hypoxia alone cause tissue injury; after ligation, superimposed hypoxia results in progressive ipsilateral ischemia (52). The threshold duration of 8% O_2 exposure that results in tissue injury is close to 1.5 hours. Overall, the severity of injury then increases with increasing duration of hypoxia. However, in this preparation, for unexplained reasons, there is considerable variability in the severity and histopathologic features of injury in lesioned animals. In this perinatal model, the propensity for selective CA1 pyramidal cell damage which characterizes ischemic brain injury in adult models, is not observed. The features of hippocampal histopathology are somewhat variable; of note, dentate gyrus neurons (considered relatively resistant to ischemic injury in adult brain) are often injured.

Alterations in endogenous EAA. In this perinatal stroke model, the first evidence that focal ischemic brain injury disrupted the functional integrity of EAA synapses was the observation that in target areas for irreversible ischemic injury (striatum and hippocampus) high-affinity glutamate uptake was acutely (and reversibly) suppressed during the evolution of injury. The importance of this finding was that it suggested a credible pathophysiologic mechanism that could account for acute accumulation of EAA in the synaptic cleft (53).

Subsequent studies using *in vivo* microdialysis provided more direct evidence that EAA accumulated in extracellular fluid (ECF) acutely with hypoxic-ischemic injury. In brain, ECF accumulation of neurotransmitters reflects regional synaptic concentra-

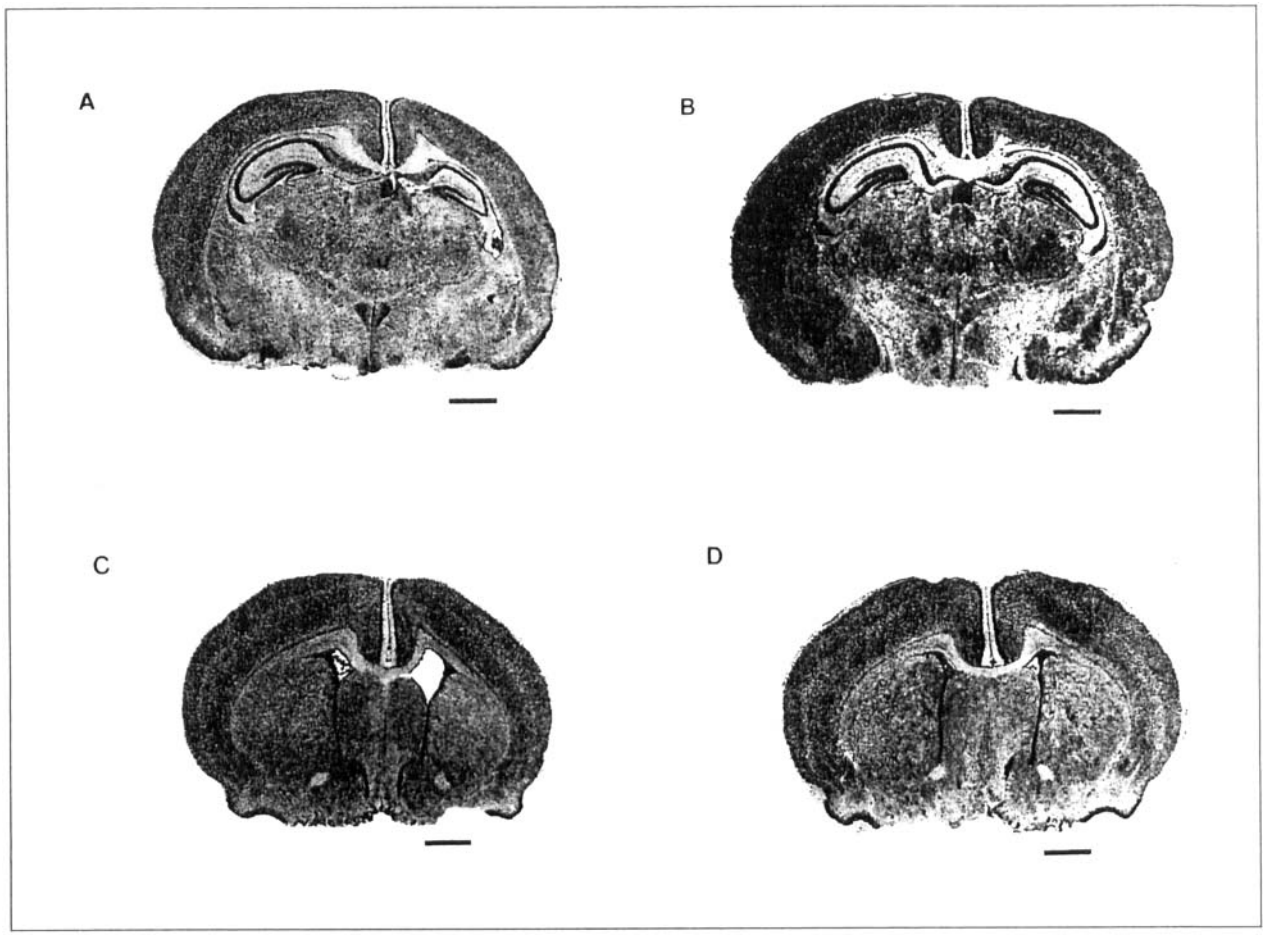

Figure 2 Glutamate antagonist neuroprotection: Both animals were subjected to right carotid ligation followed by three hours in 8% oxygen on PND 7. Brains were removed on PND 12, sectioned and stained with Cresyl violet. Sections in panels B and D are from an animal pretreated with MK-801 1 mg/kg; sections in panels A and C are from a vehicle-treated control. Coronal-sections in panels A and B are at the level of the dorsal hippocampus; sections in panels C and D are at the level of the striatum. Note the reduction in size of the right hippocampus and striatum in the control animal (panels A,C), which is not discernible in the MK-801 pretreated animal (panels B,D). Scale bar - 1.6 mm.
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tions. Although accurate determination of concentrations of amino acids in interstitial fluid is extremely complex, with microdialysis, sequential changes in ECF content of amino acids can be readily estimated within defined brain regions. Synaptic accumulation of EAA could result from increased release (from neurotransmitter or metabolic pools) or suppression of neuronal and glial re-uptake. Calculated efflux rates provide estimates of regional ECF content of compounds of interest (e.g., for striatal glutamate 1-2 μm).

Using microdialysis, Hagberg et al. found that during acute global ischemia cortical and striatal extracellular fluid glutamate and aspartate concentrations rose markedly in fetal sheep (6). We developed a method for microdialysis in striatum and hippocampus of postnatal day 7 rats, and studied amino acid efflux in the perinatal rodent focal ischemia model. Striatal ECF amino acid efflux was stable over 3 to 4 hours of sampling, and neither exposure to 8% oxygen or carotid ligation alone altered efflux (54). During the evolution of hypoxic-ischemic injury, increases (>3-fold) in striatal glutamate efflux were detected, but the patterns differed from findings in adult brain. Glutamate efflux peaks were transient, and their timing varied (54). In contrast, in an adult rodent middle cerebral artery occlusion stroke model, striatal ECF glutamate concentration increased to >60 times baseline 1 to 3 hours later (55). In hippocampus, using a slightly different experimental protocol, we found a progressive rise in glutamate efflux with increasing duration of hypoxia; the time of maximal efflux coincided with the threshold for ischemic injury and values returned to baseline immediately post-hypoxia (56). To facilitate more detailed comparison of sequential changes in glutamate and glutamine efflux, for each animal, values were normalized, and expressed as a percentage base-

line (averaged from first three fractions) (Figure 1). Mean glutamate efflux peaked early in the second hour of hypoxia while glutamine efflux declined progressively. In contrast with glutamate, glutamine efflux declined gradually; the diverging trends suggest that systemic derangements such as dehydration were unlikely to account for the increase in glutamate efflux observed. The significance of the glutamine decline is uncertain; inhibition of the glial enzyme glutamine synthetase, which converts glutamate to glutamine, could account for both a reduction in glutamine efflux and synaptic glutamate accumulation (57). Awareness of the role of endogenous glycine in potentiating activation of NMDA receptors (58) prompted interest in analysis of glycine release; although no significant change in hippocampal glycine efflux was detected, our data revealed a trend towards increased glycine efflux in the second hour of hypoxia (56). Other investigators, using somewhat different dialysis methods in the same animal model, found that in cerebral cortex, ECF concentrations of glutamate and glycine rose acutely in hypoxic-ischemic cortex, and that while glutamate concentrations return to normal post-hypoxia, glycine concentrations remain elevated (59).

In adult brain, aspartate efflux generally increases in parallel with changes in glutamate efflux during ischemia (5,60); however, we found no consistent increase in hippocampal aspartate efflux. This difference likely reflects lower synaptic concentrations of aspartate in immature brain; whether this developmental difference in EAA metabolism has pathophysiologic significance as to mechanisms of neuronal injury is unknown.

It is important to acknowledge evidence suggesting that increased extracellular glutamate concentrations alone are not sufficient to produce brain injury (61,62). In the adult brain, the balance between excitatory and inhibitory amino acid neurotransmitter concentrations may correlate better with injury than extracellular glutamate alone (62). Of interest, in contra-distinction with studies in adult brain, baseline hippocampal GABA efflux was often undetectable in immature rodents, and no increases were detected with hypoxia-ischemia. Immaturity of GABAergic innervation may also be a contributing factor to the pathophysiological differences in the evolution of ischemic neuronal injury between immature and adult brain. Factors such as intrinsic variation in cellular susceptibility to excitotoxic injury and regional activity of endogenous neuromodulatory compounds are also likely to play critical roles in determining the extent of ischemic neuronal damage.

Neuroprotection with EAA antagonists. The most convincing evidence that EAA contribute to the pathogenesis of perinatal hypoxic-ischemic brain injury comes from neuroprotection studies with EAA antagonists. Several investigators have demonstrated that the non-competitive NMDA antagonist MK-801 protects the immature rat brain from focal hypoxic-ischemic injury. McDonald et al. (7) found that in seven day old rats that underwent right carotid ligation and were treated with 1 mg/kg MK-801 immediately before or 75 minutes after the onset of hypoxia (three hours total duration, in 8% oxygen) the severity of brain injury was reduced, assessed by comparison of cerebral hemisphere weights five days later, as compared with untreated litter-mates. The same treatment also attenuated the acute ipsilateral loss of EAA receptor binding observed 24 hours post-hypoxia in hippocampus and striatum (63). MK-801 administered immediately before or one hour into hypoxia (two hours total duration, in 8% oxygen) also prevented post-hypoxic-ischemic impairment in a learning and memory task at 30-days age and prevented hippocampal CA1 and CA3 pyramidal neuronal loss as assessed at 60-days age (64).

Figure 2 compares the distribution of histopathology in two animals that underwent carotid ligation and hypoxic exposure on PND 7, one of which was treated with MK-801; in the untreated animal, sacrificed on PND 12, there is prominent unilateral forebrain injury, with marked substance loss in striatum hippocampus, and cortex. In this MK-801-treated animal there was considerable attenuation of injury; however, it is important to consider that there is considerable variability in the efficacy of MK-801 against hypoxic-ischemic injury in this model. In contrast, the same dose of MK-801 (1 mg/kg) consistently completely prevents pure NMDA-induced injury (65).

In a closely related injury model, in which low atmospheric pressure was combined with unilateral carotid ligation to elicit focal hypoxic-ischemic brain injury in the immature rat, treatment with MK-801 1 mg/kg, prior to hypobaric exposure, markedly decreased the severity of ipsilateral brain damage (66). In a model more closely approximating global perinatal cerebral hypoxia-ischemia (bilateral carotid ligation plus one hour in 8% oxygen), Hattori et al. (67) demonstrated attenuation of cortical and striatal damage by administration of MK-801 10 mg/kg shortly after hypoxia, and complete prevention of damage in survivors if the same high dose was administered immediately before hypoxia (but with increased acute mortality).

In adult animals, it has been suggested that the beneficial effects of MK-801 were attributable to MK-801-induced hypothermia (18). In the hypobaric-ischemia model, a mild elevation in body temperature was associated with MK-801 treatment (66); neither McDonald et al. (7) or Ford et al. (64) reported body or brain temperature measurements. However, when

temperature was monitored in unlesioned seven day old rats after treatment with the same dose (1 mg/kg) of MK-801, only slight reductions were observed (0.98 ± 0.08 °C, n=4) (68), and in lesioned seven day old rats pretreated with MK-801, 10 mg/kg, rectal temperature over a 24 hour period did not differ from controls (69). Thus, in immature rodents, hypothermia cannot account for neuroprotection.

The efficacy of other EAA antagonists has also been tested in the same model. Treatment with kynurenate, which blocks both NMDA and non-NMDA receptors, prior to hypoxia, reduced cerebral edema (70); administration of the same dose of kynurenate after hypoxia-ischemia attenuated ipsilateral hemisphere weight loss, an accurate measure of the severity of brain injury (71). Similarly, treatment with dextromethorpan, another non-competitive NMDA antagonist, prior to hypoxia, reduced the incidence of frank ipsilateral cerebral infarction (72).

A recent study in a model of incomplete global cerebral ischemia in the piglet yielded different results; MK-801 provided incomplete neuroprotection, limited to the hippocampus (73). Differences in the pathophysiology of ischemic injury (e.g., global or focal), and drug metabolism could account for such discrepancies in neuroprotective efficacy in different experimental paradigms.

No published studies have examined the neuroprotective effficacy of AMPA antagonists such as NBQX in perinatal ischemic brain injury. Optimal protective regimens for cerebral ischemia may require blockade of both NMDA and non-NMDA receptors (74), and such combination therapies have not yet been evaluated.

Conclusions

Thus, in perinatal rodent brain there is strong evidence that EAA play a major role in the pathogenesis of focal ischemic injury. To what extent these observations are relevant to treatment of ischemic brain injury in human infants remains uncertain. In considering treatment with EAA antagonists, an important issue that must be acknowledged is their potential detrimental effects in the developing brain. Since EAA play critical roles in normal synaptic maturation, EAA blockade could disrupt normal developmental processes, and the adverse impact of EAA antagonists will require careful evaluation before such drugs come into use clinically. Drugs that blocked excessive EAA release, or enhanced re-uptake might provide neuroprotection more selectively than receptor antagonists. Another potentially important pharmacologic strategy that must be explored is the development of combination therapies, targeting multiple components of the complex biochemical cascade that leads to irreversible ischemic injury, including EAA receptor over-activation, concurrently.

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